Engineering temperature-sensitive SH3 domains

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Background: The ability to control specific protein–protein interactions conditionally *in vivo* would be extremely helpful for analyzing protein–protein interaction networks. SH3 (Src homology 3) modular protein binding domains are found in many signaling proteins and they play a crucial role in signal transduction by binding to proline-rich sequences.

Results: Random *in vitro* mutagenesis coupled with yeast two-hybrid screening was used to identify mutations in the second SH3 domain of Nck that render interaction with its ligand temperature sensitive. Four of the mutants were functionally temperature sensitive in mammalian cells, where temperature sensitivity was correlated with a pronounced instability of the mutant domains at the nonpermissive temperature. Two of the mutations affect conserved residues in the hydrophobic core (Val133 and Val160), suggesting a general strategy for engineering temperature-sensitive SH3-containing proteins. Indeed mutagenesis of the corresponding positions in another SH3 domain, that of Crk-1, rendered the full-length Crk-1 protein temperature sensitive for function and stability in mammalian cells.

Conclusions: Construction of temperature-sensitive SH3 domains is a novel approach to regulating the function of SH3 domains *in vivo*. Such mutants will be valuable in dissecting SH3-mediated signaling pathways. Furthermore, the methodology described here to isolate temperature-sensitive domains should be widely applicable to any domain involved in protein–protein interactions.

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Introduction

Temperature-sensitive mutations, which render the function of a protein dependent on the growth temperature of cells harboring the mutant, have proved to be powerful tools in establishing the role of a protein *in vivo*. Temperature-sensitive mutants have been used with great success for many years in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The flexibility of yeast genetics and the wide range of growth temperatures, typically 24–37°C, explain this success. However, mammalian cells can also be grown over a range of temperatures (between 32 and 41°C), and with the development of polymerase chain reaction (PCR) techniques, random mutagenesis of vertebrate genes can be accomplished quickly and easily. The potential of this approach is illustrated by the important historical role played by temperature-sensitive mutants of oncogenes and tumor suppressor genes. For example, a temperature-sensitive mutant of p53 (mouse Val135→Ala, corresponding to human Val138→Ala) shed light on its role as a tumor suppressor and inducer of apoptosis [1–3]. Interestingly, several tumor-derived mutant p53 proteins displayed a temperature-sensitive DNA binding capability, suggesting that the destabilization of the three-dimensional folding could be one mechanism of oncogenesis [4]. Temperature-sensitive mutants of the Rous sarcoma virus oncogene Src were invaluable in demonstrating that v-Src transforming activity was independent of viral replication [5] and that its tyrosine kinase activity was correlated with cell transformation [6].

Src homology 3 (SH3) modular protein-binding domains function as molecular adhesives, and they are found in a wide variety of signaling proteins, including protein and lipid kinases, protein phosphatases, phospholipases, Ras-controlling proteins and adaptor proteins [7,8]. Through their binding to specific proline-rich sequences, SH3 domains are known to play a crucial role in the formation of the multiprotein complexes responsible for signal transduction. Although it is relatively easy to identify potential SH3 binders *in vitro*, it is very difficult to demonstrate the biological significance of a particular SH3-mediated interaction *in vivo*. Overexpression, gene knock-outs, loss-of-function and dominant-negative mutants are all useful approaches that are being currently exploited. All these approaches are hampered by their constitutive nature, however; for example, immediate effects can be obscured by adaptation mechanisms, and overexpression can have toxic effects that preclude isolation of expressing clones. As a new strategy to overcome this difficulty, we chose to develop a temperature-inducible system to control SH3-dependent interactions.

As a model system we chose the well-characterized interaction between the SH3 domain of the Nck adaptor and

the Pak1 kinase [9–11]. Nck consists of three SH3 domains (SH3-1, SH3-2 and SH3-3) followed by an SH2 domain [12], suggesting that it can function to mediate complex formation in response to tyrosine phosphorylation events, relocalizing SH3-binding effector proteins to sites of tyrosine phosphorylation. Very little is known, however, about its biological role and the specific signaling pathway(s) in which it is involved. Different approaches have identified several binding partners for Nck, but the physiological significance of these interactions has been difficult to demonstrate. Different groups have showed that Nck interacts via the second of its three SH3 domains (SH3-2) with the first proline-rich motif of the Pak1 serine/threonine kinase [9–11]. The Nck/Pak1 interaction can mediate the membrane relocalization and subsequent activation of the Pak1 kinase [11]. Pak1 is known to be activated by members of the Rho family of small GTPases, such as Cdc42 and Rac1 [13–15], and to be involved in cytoskeleton and focal adhesion reorganization [16–19].

Other SH2/SH3 adaptors include Grb2 and Crk and their relatives. Grb2, which consists of one SH2 domain between two SH3 domains, couples tyrosine-phosphorylated membrane proteins, such as the growth factor receptors, with the guanine nucleotide exchange factor Sos, which is an activator of the small GTPase Ras [20]. Crk, originally isolated as a viral oncogene [21], exists in two alternate splicing forms. Crk-1 contains one SH2 domain followed by an SH3 domain. Crk-2 has an additional carboxy-terminal SH3 domain [22]. Overexpression of Crk-1 increases the cellular level of tyrosine-phosphorylated proteins and can weakly transform fibroblasts [22], but the signal pathways involved are still unclear.

In this study we describe a novel mutagenic two-hybrid screen to generate temperature-sensitive protein–protein interaction domains. We report the identification of temperature-sensitive mutations for the Nck SH3-2 domain, and further demonstrate that the corresponding mutations in the SH3 domain of Crk-1 render that protein temperature sensitive. This approach will provide valuable tools for dissecting the SH3-dependent protein–protein interactions involved in signal transduction.

Results

Screening for mutations in Nck SH3-2 that are temperature sensitive for interaction with Pak1

It had already been reported that full-length Nck interacts with full-length Pak1 in the yeast two-hybrid system [9]. We restricted the size of Nck and Pak1 to the specific sites of interaction: the second SH3 domain of Nck and the amino-terminal proline-rich region of Pak1 [9,10]. The region encoding Nck SH3-2 (amino acids 106–166) was cloned into pACT2 to express this domain fused to the activator domain of the Gal4 protein. The 27 amino-terminal residues of Pak1, containing the Nck SH3-2 binding site, were fused to the Gal4 DNA-binding domain in the pAS2 vector. It was expected that the Nck/Pak-mediated interaction would reconstitute the Gal4 transcription activator and lead (in the Y190 strain) to the transcription of the two reporter genes, HIS3 and lacZ. Accordingly, the double transformants pACT2-NckSH3-2/pAS2-Pak1Nter were able to grow in the absence of histidine and produced a blue color upon β-galactosidase assay (not shown). To confirm the specificity of the interaction, we verified that the Trp143→Lys point mutation, known to destroy the binding of SH3-2 [23], abolished the induction of the reporter genes (not shown).

DNA encoding the Nck SH3-2 domain was randomly mutagenized *in vitro* by PCR and inserted into the pACT2 vector directly using *in vivo* recombination in the Y190 + pAS2-Pak1Nter strain (see the Materials and methods section). Using this method we obtained a pool of yeast clones co-expressing Pak1Nter and randomly mutated SH3-2. We screened for clones displaying a Nck/Pak interaction that was dependent on temperature by replica plating the colonies at 25°C and at 33°C. The choice of the restrictive temperature was limited by the inability of the Y190 double transformants to grow at temperatures higher than 33°C in selective medium. A total of 3×10^4 colonies were screened, and 60 were isolated that could grow without histidine and produce β-galactosidase at 25°C, but not at 33°C. The pACT2-NckSH3-2 plasmids were isolated from the 60 yeast clones, amplified in *Escherichia coli* and re-transformed in the Y190+ pAS2-Pak1Nter strain to confirm the temperature-sensitive phenotypes. Of the 60 clones, 16 retained a temperature-dependent Nck/Pak interaction and the mutant SH3-2 genes were sequenced.

Sequencing identified nine single substitutions and three double mutations in the Nck SH3-2 domain (Figure 1). In three cases the same point mutation was found in two independent clones. One sequence was apparently wild type, so the temperature-sensitive phenotype in this case must have been the result of unknown modification outside the SH3 domain. The most common type of substitution was a change of the charge of the sidechain (for example lysine→glutamine), which occurred in seven out of 15 substitutions. In four cases an amino acid with a hydrophobic sidechain was changed to a smaller hydrophobic residue (for example valine→alanine). Taking advantage of the known three-dimensional structure of the SH3 domain of the proto-oncogene tyrosine kinase c-Abl [24], we verified that two of these mutations affect residues in the hydrophobic core: Val133→Ala and Val160→Ala of Nck. The other substitutions are located in the loops connecting the five β strands, and only one mutation (Glu122→Lys) is found in the region predicted to contact the left-handed polyproline helix of the ligand.

Figure 1

Sequences of the temperature-sensitive Nck SH3-2 domains obtained from the two-hybrid screen. Mutated residues (compared with wild-type human Nck SH3-2) are highlighted in orange. The figure summarizes for all the mutants the results of the biological assay illustrated in Figure 2. The round morphology phenotype was roughly scored using the following criteria: +++, >90% round cells; ++, 75–90% round; +, 25–75% round; +/–, 10–25% round; –, <10% round.

Biological assay for the temperature sensitivity of Nck SH3-2 mutants

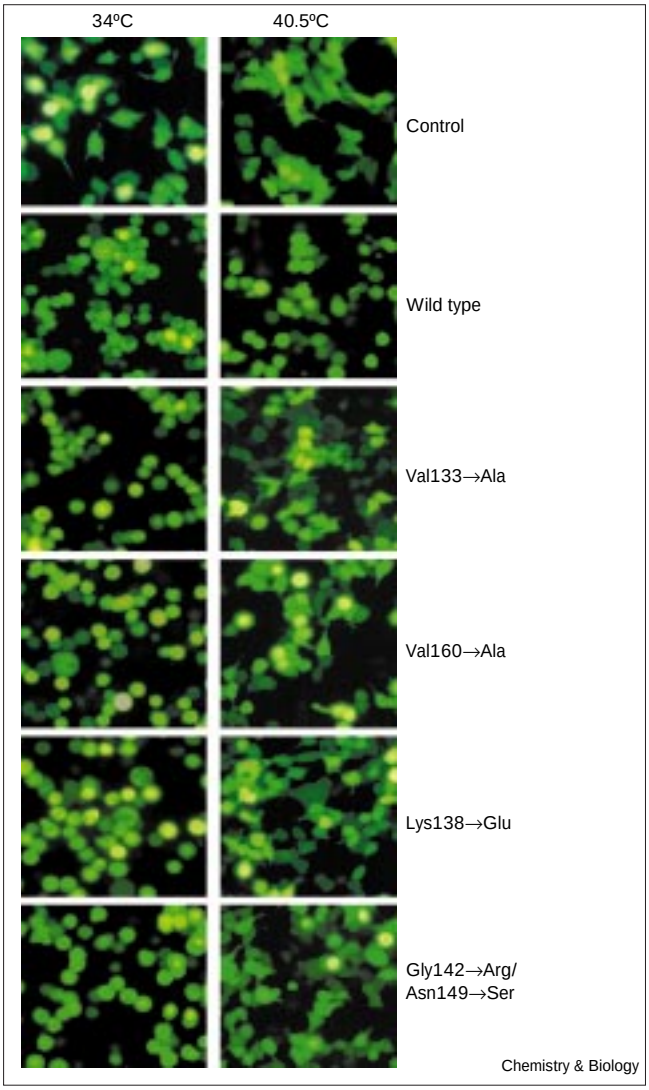
We next tested the functionality of the mutants identified via the yeast two-hybrid system in a mammalian cell culture system. We had previously observed that membrane localization of Nck SH3-2 via fusion to a myristoylation signal induces a phenotypic change in 293T human kidney cells: the cells become round and loosely attached to the dish, suggesting some cytoskeletal modifications [11]. This provided a quick morphological read-out to test the temperature sensitivity of the Nck SH3-2 mutants *in vivo*.

293T cells were transiently transfected with vectors expressing a myristoylated, hemagglutinin (HA)-tagged form of the Nck SH3-2 mutants, along with pEBB-GFP (green fluorescent protein) to identify the transfected cells, and morphology was examined (the results are summarized in Figure 1). Two days after transfection, all 12 mutant cell lines, as well as the wild-type myristoylated Nck SH3-2, had very round morphology at 34°C, indicating the SH3 domains were functional at this low temperature. At 40.5°C, in contrast, cells expressing four of the mutants (Val160→Ala, Val133→Ala, Lys138→Glu and Gly142→Arg/Asn149→Ser) had normal morphology, comparable to those expressing vector alone or unmyristoylated wild-type NckSH3-2, indicating that these SH3 domains do not function at high temperatures (Figure 2). The wild-type myrNckSH3-2 and to varying extents the other eight mutants induced a round morphology even at high temperature. The phenotypic behavior of the four temperature-sensitive mutants was roughly equal, even at the intermediate temperature of 37°C, at which their morphological changes were modest. It is interesting that all 12 mutants were functional at 34°C and eight of them even at 40.5°C; because in the yeast two-hybrid screening the restrictive temperature was 33°C, the Nck SH3-2 domain was more stable in the mammalian cellular environment than in yeast. This may be explained by the presence of various SH3-binding partners in mammalian cells or by more effective chaperones.

To verify that temperature was indeed able to control the phenotype of cells transfected with mutant myrNckSH3-2, we performed reciprocal temperature shift experiments. Cells were grown at 40.5°C or 34°C for 24 h after transfection and then shifted to 34°C or 40.5°C, respectively, for an additional 24 h. The cells clearly adapted their morphology as a function of the growth temperature, showing that the morphological changes are reversible (data not shown). Taken together, these results show that the mutations Val160→Ala, Val133→Ala, Lys138→Glu and Gly142→Arg/Asn149→Ser in the SH3-2 of Nck render the function of this domain temperature sensitive in mammalian tissue culture cells.

We next determined whether the temperature-sensitive mutations affected the level of the proteins by western blot analysis of whole 293T cell lysates (Figure 3a). At 34°C the level of the Val160→Ala, Val133→Ala, Lys138→Glu forms of myrNckSH3-2 was comparable to the wild type, and the

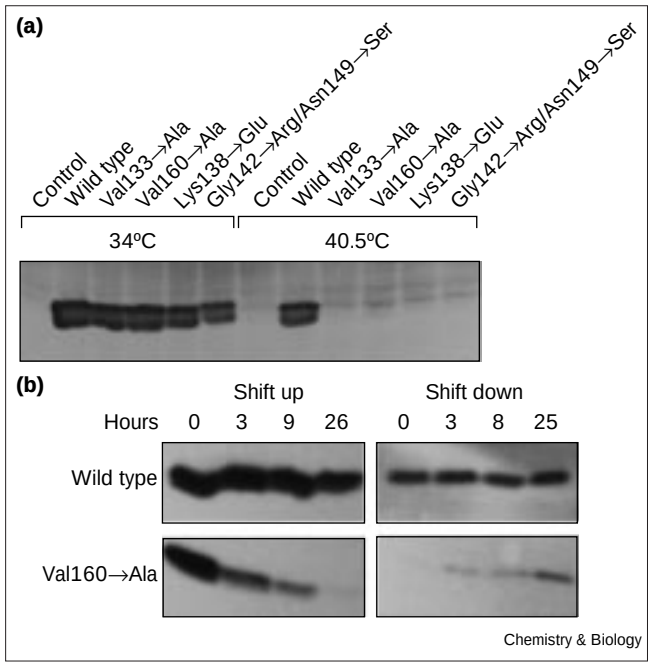
Figure 2



Morphology of 293T cells transfected with myrNckSH3-2 and its temperature-sensitive derivatives. Cells were transfected in duplicate at 37°C with pEBB vectors (4 μg per 10 cm dish) expressing the various myrNckSH3-2 constructs. A pEBB vector expressing GFP (2 μg) was cotransfected to visualize the morphology of the transfected cells only. After 8 h the medium was changed and cells were further incubated at 34°C or 40.5°C. Photomicrographs of living cells were taken 2 days after transfections under UV illumination. Typically the transfection efficiency was 50–80%. Successfully transfected cells are green because of GFP expression; the dark areas represent nontransfected cells.

Gly142→Arg/Asn149→Ser mutant was slightly decreased. At 40.5°C the level of all four temperature-sensitive mutants was greatly decreased, to the point that they were barely detectable. Kinetic studies showed that the level of protein significantly decreased a few hours after shift to the nonpermissive temperature and was minimal after one day. Conversely, following a shift down to the permissive temperature, the mutated products began to accumulate

Figure 3



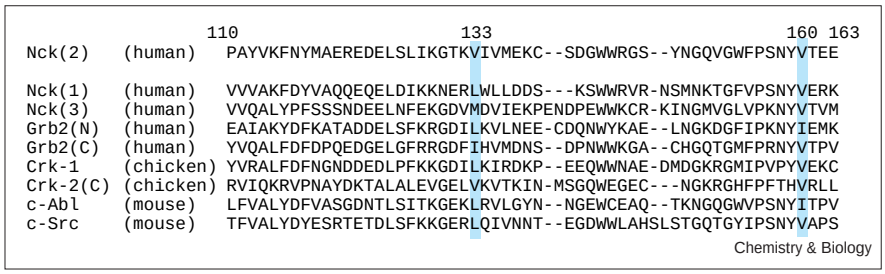
Protein levels of wild-type and temperature-sensitive myrNckSH3-2 at different temperatures. (a) 293T cells were transfected as described in Figure 2 and lysates prepared 2 days after transfection. (b) After transfection the cells were incubated for 2 days at 34°C or 40.5°C. On the first day the cells from 10 cm dishes were digested with trypsin and replated into 6-well dishes. On the second day the 6-well dishes were reciprocally shifted. Shift up: 34°C→40.5°C; shift down: 40.5°C→34°C. Lysates were prepared at the indicated times after shift. Equal amounts of total proteins were loaded on 15% SDS polyacrylamide gels and the myrNckSH3-2 products were detected by western blots probed with anti-HA antibodies.

in a few hours and took one day to reach a high level (Figure 3b). The most probable interpretation of these results is that the folded structure of the mutated domains is relatively unstable at the restrictive temperature, and consequently the mutant domains are more susceptible to proteolytic degradation. We cannot rule out the hypothesis that the mutations also affect the biosynthesis of the mutated myrNckSH3-2, however.

Design of a temperature-sensitive c-Crk-1 SH3 domain

Alignment of the sequences of various SH3 domains showed that two of the mutations that render the SH3-2 domain of Nck temperature sensitive are in highly conserved residues. At the positions corresponding to residues 133 and 160 of Nck virtually all known SH3 domains have large hydrophobic residues such as leucine, valine or isoleucine. Figure 4 shows an alignment of the SH3 domains of the adaptor proteins (Nck, Grb2 and Crk) and of the nonreceptor kinase oncogenes c-Abl and c-Src. The sidechains of residues 133 and 160 are located into the hydrophobic core of the SH3 domain [24], so they are expected to play a structural role in stabilizing the folded

Figure 4



Two temperature-sensitive mutations affect conserved residues of the SH3 domain hydrophobic core. Alignment of the second SH3 domain of Nck with the sequences of other SH3 domains. Nck (1), (2) and (3) are the first, second and third SH3 domains of human Nck, respectively. Grb2 (N) and (C) are the amino-terminal and carboxy-terminal SH3 domains of Grb2. Crk-1 and Crk-2 originate from alternative splicing; the amino-terminal SH3 domain of Crk-2 is identical to the only SH3 domain of Crk-1, and Crk-2(C) is the carboxy-terminal SH3 domain of Crk-2. The residues corresponding to Nck residues Val133 and Val160 are highlighted in blue.

conformation. Moreover, in our mutants the temperature-sensitive effect resulted from valine→alanine substitutions at these residues, as in the case of the temperature sensitive mutation in p53 [1]. It is not surprising that the resulting 'hole' in the hydrophobic core of a small domain could destabilize the folded structure. We reasoned that analogous mutations reducing the size of hydrophobic sidechains at these positions might have the same temperature-sensitive effect on the stability of the SH3 domains of other SH3-containing proteins.

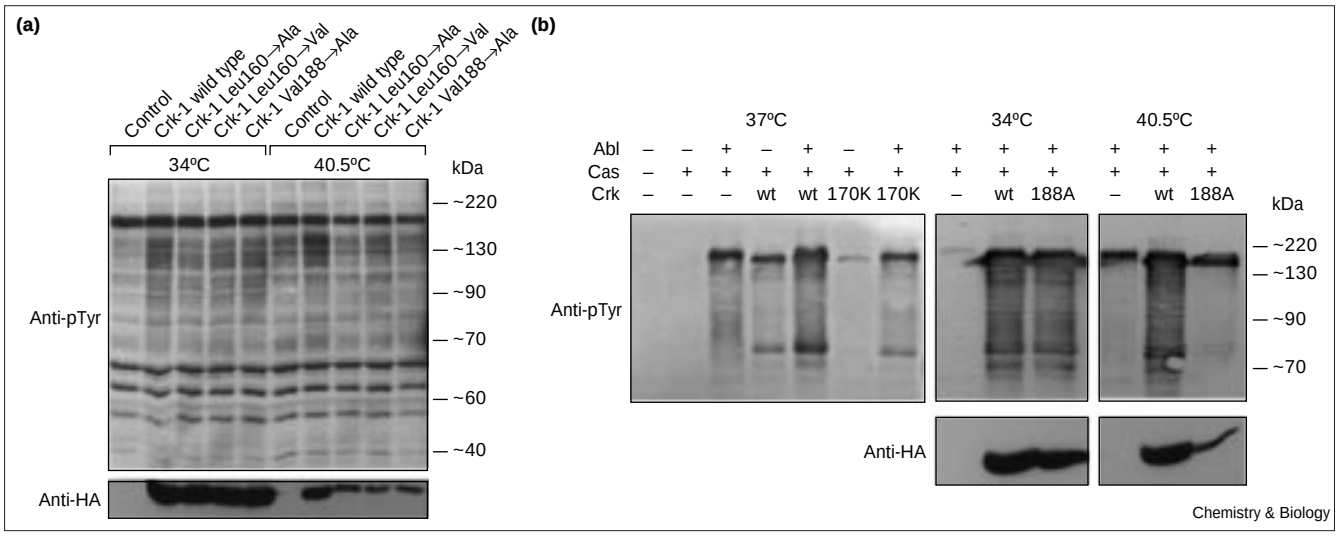
To test this hypothesis we modified Crk-1, an adaptor protein that consists of an amino-terminal SH2 domain and a single SH3 domain. We introduced the substitutions leucine→alanine and leucine→valine at Crk-1 position 160 (corresponding to position 133 of Nck) and valine→alanine at Crk-1 position 188 (corresponding to position 160 of Nck). 293T cells were transiently transfected with vectors expressing HA-tagged wild-type Crk-1, Crk-1 Leu160→Ala, Crk-1 Leu160→Val and Crk-1 Val188→Ala. Overexpression of Crk-1 is known to increase the tyrosine phosphorylation of several proteins [22], and this has been shown to require both the SH2 and SH3 domains in the case of v-Crk [25]. We therefore measured the level of tyrosine-phosphorylated proteins in total lysates to assay the function of the various Crk-1 products. At 34°C, overexpression of all Crk-1 forms stimulated the tyrosine-phosphorylation of various proteins particularly in the 100–150 kDa range, with the Leu160→Ala mutant being slightly less effective because of the stronger nature of this substitution. At 40.5°C the wild-type form increased the level of tyrosine-phosphorylated proteins relative to the control, whereas the three mutants did not (Figure 5a). We observed in this and other experiments that the basal level of tyrosine phosphorylation is generally higher at 40.5°C than at 34°C. We also found that the three mutations decreased the stability of the Crk-1 protein at 40.5°C, whereas at 34°C the mutants displayed the same protein levels as the wild type (Figure 5a), demonstrating that a temperature-sensitive mutation in a single domain can compromise the stability and function of a larger protein containing it.

The major tyrosine-phosphorylated protein in Crk-transformed cells is p130Cas (for Crk-associated substrate) [26]. Studies *in vitro* with purified components have showed that p130Cas is an excellent substrate for the c-Abl kinase and that Crk-1 strongly enhances the ability of Abl to hyperphosphorylate p130Cas [27]. The SH3 domain of Crk-1 is required for this function [27], consistent with the Abl–Crk interaction being mediated by the Crk SH3 domain [28,29]. We coexpressed in 293T cells the substrate p130Cas (as a GST fusion protein) together with c-Abl and either wild-type or Val188→Ala Crk-1. p130Cas hyperphosphorylation was detected by anti-pTyr western blot of total cell lysates. In addition to the full-length band, the phosphorylation of p130Cas appeared as a smear of more slowly migrating forms and of lower bands because of the large amount of p130Cas and the presence of many truncated forms. The presence of the smear was a convenient marker of the extent of phosphorylation of p130Cas. The Trp170→Lys Crk-1 mutant, whose SH3 binding capability is destroyed [23], did not facilitate hyperphosphorylation of p130Cas, confirming in our system that a functional SH3 domain is required (Figure 5b). At 34°C Crk-1 Val188→Ala was as effective as the wild-type protein in promoting Abl-induced hyperphosphorylation of p130Cas, whereas at 40.5°C this mutant was completely ineffective (Figure 5b). Once again, the loss of function correlated with a reduced stability (Figure 5b).

Discussion

Understanding complicated intracellular protein–protein interaction networks is one of the major challenges in signal transduction. Adaptor proteins like Nck and Crk play a key role in establishing the multiprotein complexes responsible for cellular responses to extracellular signals. Although the model of their action (relocalization of SH3-binding partners to SH2-binding sites) is conceptually simple, it is very difficult to unambiguously disentangle the molecular events responsible for the output of a particular signal. As one novel approach to help solve these problems, we report here the development of an inducible system in which SH3-dependent interactions are temperature dependent.

Figure 5



Function and protein levels of engineered temperature-sensitive Crk-1 at different temperatures. (a) 293T cells were transfected in duplicate at 37°C with vectors expressing wild-type Crk-1 or Leu160→Ala, Leu160→Val, or Val188→Ala mutants (4 μg per 10 cm dish). After 8 h the medium was changed and the cells were maintained at 34°C or 40.5°C as indicated. Lysates were prepared 2 days after transfections. Equal amounts of total protein were loaded on SDS polyacrylamide gels, blotted and probed with a mixture of 4G10 and PY20 monoclonal anti-pTyr antibodies, or anti-HA antibodies to detect Crk-1 proteins. (b) 293T cells were transfected at 37°C with pBPN-Abl, pEBG-p130Cas and pEBB-Crk-1 (wild type, Trp170→Lys or Val188→Ala) as indicated (4 μg each vector per 10 cm dish). After 8 h dishes were incubated at different temperatures as indicated and lysates were prepared and analyzed as in (a). Approximate positions of molecular weight standards are indicated to the right.

The two-hybrid system has been extensively used to detect interactions between proteins, and it offered us a quick and efficient way to screen for temperature-sensitive mutations in an SH3 domain. We identified four mutations in the second SH3 domain of Nck that conferred temperature sensitivity; the function of the domain could be switched on and off *in vivo* simply by changing the growth temperature of the cells. As the screening method is straightforward and based on well-established techniques, it could easily be applied to identify temperature-sensitive mutations for other protein–protein interactions. For this purpose it would be very useful to isolate yeast strains, genetically suitable for the two-hybrid system, that sustain growth at high temperatures such as 38–39°C. Our screening was limited to a maximum non-permissive temperature of 33°C, and out of 12 mutations temperature-sensitive in yeast, four were temperature-sensitive in mammalian cells. It seems likely that the availability of yeast strains that are viable at higher temperatures will increase the correlation between the yeast and mammalian systems. This basic strategy is likely to be generally useful in a wide range of contexts to isolate temperature-sensitive protein-binding domains.

At the molecular level, temperature-sensitive mutations generally destabilize the folded state by destroying specific intramolecular interactions [30]. Two mutations (Val133→Ala and Val160→Ala) that emerged from our screen affected the hydrophobic core of the SH3 domain, suggesting they work by weakening the structure of the module rather than by directly influencing protein–protein interactions. The conservation of these two positions in almost all the known SH3 domains raises the appealing possibility that similar mutations might render any SH3 domain of interest temperature-sensitive, without the need to screen each time for specific temperature-sensitive mutations. Indeed we proved that identical (valine→alanine) or similar substitutions (leucine→alanine and leucine→valine) reducing the sidechain hydrophobicity of the residues at these two critical positions in the SH3 domain of Crk-1 rendered the protein functionally temperature sensitive. The fact that these substitutions could make two different SH3 domains heat-labile suggests that the same approach could work with many, if not all, SH3-containing proteins. These two 'hot spots' for the thermal stability could be used to engineer temperature-sensitive SH3 domains in the same way that specific point mutations of functionally important residues have been used to destroy binding activity or enzymatic activity of other conserved domains.

Changes in protein level and cell morphology are relatively long-term effects; we were also interested in measuring the kinetics of activation and inactivation of the Nck SH3-2 domain in the short term after temperature shift. To this end, we examined the myrNckSH3-2-dependent activation

of Pak1 kinase activity [11]. To our surprise, however, we found that the myrNckSH3-2-dependent activation of Pak1 was inhibited very rapidly after a shift to high temperature even when the wild-type version of Nck SH3-2 was used (data not shown). This inhibition was not observed with a constitutively active mutant of Pak1 (T423E [17]) or when Pak was activated by co-expression of activated Cdc42. Moreover, this inhibitory effect did not depend on the JNK and p38 stress kinase pathways because we verified that these pathways were not activated by our temperature shift conditions (data not shown). It is possible that higher temperatures affect the regulation of Pak1 by biologically active lipids [31]. Alternately, temperature could influence the GDP/GTP ratio or localization of endogenous Cdc42 and/or Rac, which are required for activation of Pak by membrane localization [32].

Because of the lack of a short-term readout, it was difficult to measure in our systems the kinetics of the transition period, if any, during which the domain is still present but inactive. Coimmunoprecipitation experiments to assay SH3-mediated interactions directly are unsuitable, because they require lengthy incubation of cell extracts at 4°C that are likely to permit refolding of unfolded temperature-sensitive domains. As a consequence of these difficulties, we could not definitively address whether new protein synthesis is required for the functional switch after shift of temperature.

In the case of an isolated SH3 domain, like Nck SH3-2, loss of function at the nonpermissive temperature is probably due to a combination of unfolding/denaturation and subsequent proteolysis of the domain. In the case of the larger Crk1 product, which is composed of an SH2 and an SH3 domain, the full-length protein was clearly destabilized but not completely degraded at the nonpermissive temperature; under these conditions the SH3 function was severely compromised (Figure 5), suggesting that the residual protein contained a nonfunctional SH3 domain. Moreover, we have observed that introduction of temperature-sensitive mutations into the SH3-2 domain of full-length Nck does not affect the stability of the mutant protein at higher temperature (M.C.P. and B.J.M., unpublished observations). This suggests that the presence of other domains may prevent denaturation of the full-length protein and/or targeting to cellular proteases, and that the position of the SH3 domain or the length of the protein may be critical for stability. We presume that some temperature-sensitive mutations, particularly those in solvent-accessible surface residues, might eliminate binding activity without leading to denaturation and degradation of the proteins where they reside; in the case of the Nck SH3-2 domain, however, we found that mutations affecting surface residues (Lys138→Glu and Gly142→Arg/Asn149→Ser) also led to the rapid degradation of the domain upon temperature shift.

When considering applications of temperature-sensitive protein interactions, it is important to consider that two quite distinct scenarios are possible upon shift to the nonpermissive temperature: that the binding activity of the temperature-sensitive domain is lost without affecting the other functional properties of the protein, or that the mutant proteins are destabilized and degraded. In the first case it would be possible to study the function of single domains in a larger protein, whereas in the second case the instability of temperature-sensitive proteins could be exploited to remove dominant activities.

Compared with other inducible systems, such as those in which transcription is regulated by tetracycline [33], the use of temperature-sensitive mutations offers several advantages. As no promoter regulation is involved, it should not be necessary to screen many clones to find those that induce well and have low basal activity. Moreover, the inactivation and/or degradation after shift to the nonpermissive temperature is quite rapid (Figure 3b), much faster than the gradual decay obtained following inhibition of transcription.

The availability of temperature-sensitive mutations for SH3 domains paves the way for many applications. First, it will be possible to study the early events following the activation or inactivation of a specific SH3 domain, allowing dissection of the specific pathways in which a particular SH3 function is required. Second, temperature inducibility could allow the establishment of cell lines expressing proteins and/or domains that are toxic for the cell. As one example, it is impossible to obtain cell lines expressing high levels of c-Abl because of its growth-inhibitory properties [34]. Abl genes lacking a functional SH3 domain are transforming [35,36], so if Abl mutants with a temperature-sensitive SH3 domain were available, it would be possible to establish cell lines at the nonpermissive temperature, then shift down to the permissive temperature to induce expression of high levels of 'wild-type' c-Abl. Third, as the SH3 domain has been used as model to study protein folding *in vitro* [37–39], comparison of the *in vitro* properties of the wild-type and temperature-sensitive forms and study of the transition between the folded and denatured states driven by changes in temperature may help clarify the processes that enable domains to adopt a stable conformation.

Significance

Protein–protein interactions play a critical role in signal transduction and many other cellular activities. Such interactions are often mediated by conserved protein modules such as Src homology 3 (SH3) domains. We describe here a rapid and efficient method to generate conditional alleles in protein-interaction domains where their functionality is controlled by temperature. We find that SH3 domain mutants so identified are temperature

sensitive for function and protein level in mammalian cells, that some of these mutations can also confer temperature sensitivity on heterologous SH3 domains, and that a mutant SH3 domain can functionally inactivate a larger protein containing it. We expect that this new tool will not only help clarify the biological roles of many SH3-dependent interactions that play such a crucial role in signal transduction, but also become a general tool to generate temperature-sensitive conditional mutants for a wide variety of protein-interaction domains.

Materials and methods

In vitro *random mutagenesis of Nck SH3-2*

Random mutagenesis of human Nck SH3-2 was carried out using PCR under conditions of reduced Taq polymerase fidelity [40]. The 50 μl PCR reactions contained: uncut pACT2-NckSH3-2 as DNA template (10 ng), 2 primers (0.5 μM each), 1 × reaction buffer (standard from Stratagene), 2.5 units Taq polymerase (Stratagene), $MgCl_2$ (2, 4 or 6 mM), $MnCl_2$ (0.05, 0.1, 0.3 or 0.6 mM), the four dNTPs (three at 1 mM and one at 0.2 mM). Only the reactions with higher yields, corresponding to $MgCl_2$ 4–6 mM and $MnCl_2$ 0.3–0.6 mM, were used for *in vivo* recombination (see below). Most of the mutations were isolated from reactions containing a limiting concentration of dATP and were base changes of transition type (A/T→G/C).

Plasmid constructions

Details regarding the pACT2 and pAS2 vectors for the two-hybrid system are available from Clontech. An *Nco*I–*Bam*HI fragment encoding the second SH3 domain of Nck (amino acids 106–166) was generated using PCR and cloned into the *Nco*I and *Bam*HI sites of pACT2. A PCR-generated *Nco*I–*Bam*HI fragment encoding the first 27 residues of Pak1 was cloned into the *Nco*I and *Bam*HI sites of pAS2. The mutated SH3-2-encoding regions were subcloned using PCR from pACT2 into the *Bam*HI and *Asp*718 sites of pEBB-SrcHA [11], generating Nck SH3-2 constructs with the myristoylation signal from Src at the amino terminus and the influenza virus HA epitope at the carboxyl terminus. Site-directed mutagenesis of chicken Crk-1 was carried out by overlap extension using PCR with mutant oligonucleotides [41]. The mutated *Bam*HI–*Asp*718 fragments were then cloned into the *Bam*HI and *Asp*718 sites of pEBB-HA [11]. The pEBG-p130Cas has been described [27] and the pBPN-Abl expresses wild-type c-Abl type IV via a modified version of the murine retroviral vector pBABE-puro [42].

Yeast two-hybrid system

Standard yeast protocols were used (Clontech, Yeast Protocols Handbook). *Saccharomyces cerevisiae* strain YI90 (*MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 +URA3::GAL→lacZ, LYS2::GAL(UAS)→HIS3cyh*^{r}) (from A. Dutta and S. Elledge) was transformed by the LiAc method and grown in the appropriate selective conditions, –leu for pACT2 plasmids and –trp for pAS2 plasmids. 3-amino-1,2,4-triazole (3-AT) at 20 mM was added to the medium to inhibit the leaky basal HIS3 expression in the Y190 strain. β-galactosidase activity was assayed by the colony-lift method. Plasmids were isolated from yeast using glass beads and phenol:chloroform extraction and then transformed into *E. coli* HB101 by electroporation for amplification. *In vivo* recombination of gapped pACT2 and the PCR Nck SH3-2 products was performed essentially as described previously [40]: 250–500 ng *Nco*I–*Bam*HI cut pACT2 was contransformed with 10 μl of the PCR reactions (1–2 μg). With the primers we chose for mutagenic PCR, there were 92 and 86 nucleotides of homology flanking the gap of the vector for the recombination.

Mammalian cell transfection and western blots

293T cell lines, growth conditions and calcium phosphate-mediated transient transfection have been described elsewhere [23]. The cells were lysed in 1 ml KLB buffer (25 mM Tris-HCl pH = 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin solution (Sigma A-6279), 1mM DTT and 0.1 mM H_2O_2-activated sodium pervanadate. For the western blots, the antibodies against the HA epitope were from BAbCO and the horseradish-peroxidase-conjugated secondary antibodies from Pierce. For the anti-phosphotyrosine immunoblots a mixture of 4G10 monoclonal antibodies (Upstate Biochemical) and PY20 [43] was used. The immunocomplexes were detected using the ECL chemiluminescence method (Amersham).

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